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Review article

What makes *Cryptococcus gattii* a pathogen?

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Abstract

Cryptococcosis is an invasive fungal infection of humans and other animals, typically caused by the species *Cryptococcus neoformans* in patients with impaired immunity. However, there is growing recognition of the importance of the related species *C. gattii* in causing infections in apparently immunocompetent individuals. In particular, an ongoing outbreak of cryptococcal disease in the Pacific Northwest region, which started in 1999, has driven an intense research effort into this previously neglected pathogen. Here, we discuss some of the recent discoveries in this organism from the Pacific Northwest region and highlight areas for future investigation.

Introduction

Cryptococcus gattii is a fungal pathogen of humans and other animals that can be found both as an opportunistic infection (Hagen *et al.*, 2012) and as a primary pathogen (Kwon-Chung & Varma, 2006). *C. gattii* is a haploid, encapsulated basidiomycete yeast that is widespread in soil, trees and tree hollows (reviewed in (Springer & Chaturvedi, 2010, Harris *et al.*, 2012)).

Cryptococcosis is thought to commence upon inhalation of airborne infectious propagules, such as spores or dried yeast cells, allowing the pathogen to settle in the lungs, where it can survive and proliferate within alveolar

macrophages (Fig. 1). Typical symptoms that are associated with cryptococcosis are fever, weight loss, fatigue, night sweats, cough, chest pain, headache, vomiting and neck stiffness (Phillips *et al.*, 2015). If the pathogen reaches the central nervous system, this can lead to meningoencephalitis, the most severe form of cryptococcosis, which is always lethal without rapid treatment. Interestingly, there is a predilection of *C. neoformans* for central nervous system infection and *C. gattii* for lung infection. On the other hand pulmonary (and cerebral) cryptococcomas (large inflammatory masses) are formed during infection with *C. gattii*, but not with *C. neoformans* (Mitchell *et al.*, 1995, Chen *et al.*, 2000, Galanis *et al.*, 2010, Byrnes & Marr, 2011); the latter leading instead mainly to small pulmonary lesions (Speed & Dunt, 1995, Chen et al., 2000). This might be due to higher transmigration of *C. neoformans* through brain blood barrier via the Trojan horse mechanism according to *in vitro* studies (Sorrell *et al.*, 2015).

Early reports describing patients suffering from cryptococcosis highlighted the prevalence of men over women (Chen *et al.*, 2000) which was thought due to the exposure of males to environmental sources. Recent data indicate that in fact presence of testosterone in men, but not β-estradiol in women, may influence capsule growth and reduce phagocytosis of yeast by macrophages (McClelland *et al.*, 2013, Costa *et al.*, 2015).

Interestingly, while *C. neoformans* mainly infects immunosuppressed patients, with HIV/AIDS being the most common underlying condition, *C. gattii* is considered as a primary pathogen, since it frequently infects immunocompetent and apparently healthy individuals (Speed & Dunt, 1995, Sorrell, 2001), although, recent studies suggest several factors such as smoking, oral corticosteroids usage and older age may increase the risk of infection by this species (reviewed in (MacDougall *et al.*, 2011)). Interestingly, anti-cryptococcal antibody levels are higher during *C. gattii* than *C. neoformans* infections in immunocompetent patients (Speed *et al.*, 1996) and in cats (Malik *et al.*, 1999), and thus it is possible that undiagnosed antibody deficiencies may predispose to *C. gattii* infections (Marr *et al.*, 2012). Similarly, high concentrations of

granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies can be found in the plasma of otherwise healthy HIV-negative individuals suffering from cryptococcal meningitis (Rosen *et al.*, 2013) and, interestingly, are a significant risk factor for CNS infection by *C. gattii* but not *C. neoformans* (Saijo *et al.*, 2014).

In comparison to cryptococcosis caused by *C. neoformans* which kills 650,000 immunocompromised people suffering from HIV/AIDS every year worldwide (Park *et al.*, 2009) as well as a significant additional number of organ transplant recipients (Pappas, 2013), *C. gattii* infections are rather rare, although recent studies indicate that they may be mis- or under-diagnosed (Iverson *et al.*, 2012, Tintelnot *et al.*, 2015). *C. gattii* meningitis can be cured completely in the early stages of disease (Chen *et al.*, 2012), but often the disease is misdiagnosed as tuberculosis or other bacterial/viral pulmonary infections at this stage. Antifungal treatment is mainly based on amphotericin B in combination with 5-flucytosine and/or fluconazole (Chen *et al.*, 2013) and at later stages of the disease surgery and corticosteroids may be required (Sorrell & Chen, 2010).

There are many methods to differentiate between *Cryptococcus* species. Only *C. gattii* will produce blue colonies if grown on CGB (L-canavanine, glycine and bromthymol blue) agar (Kwon-Chung *et al.*, 1982, Min & Kwon-Chung, 1986). Similarly, capsular agglutination reactions can discriminate between *C. neoformans*, which exhibits serotypes A (*C. neoformans* var. *grubii),* D (*C. neoformans* var. *neoformans)* and AD) and *C. gattii* (which is comprised of serotypes B and C (Kwon-Chung *et al.*, 1982, Franzot *et al.*, 1999, Boekhout *et al.*, 2001)). However the most reliable methods for differentiating between *Cryptococcus* species are sequence based (McTaggart *et al.*, 2011, Kwon-Chung *et al.*, 2014, Hagen *et al.*, 2015) and rely on amplified fragment length polymorphism (AFLP; (Boekhout *et al.*, 2001)), PCR and multiplex PCR fingerprinting (Meyer *et al.*, 1999, Meyer *et al.*, 2003, Ito-Kuwa *et al.*, 2007) or sequencing of intergenic spacers (IGS; (Diaz *et al.*, 2000, Diaz *et al.*, 2005)).

For most of the time that cryptococcosis has been recognized, research efforts have focused on *C. neoformans* as the dominant pathogenic species. However, in 1999 an outbreak of cryptococcosis started on Vancouver Island (British Columbia, Canada) that was later identified as being caused by *C. gattii*. This outbreak subsequently spread to mainland Canada and the northwestern part of the USA (Oregon and Washington (MacDougall *et al.*, 2007)). Although *C. gattii* was previously known to be prevalent in tropical regions (Kwon-Chung & Bennett, 1984), its abrupt appearance in the moderate climate of the Pacific Northwest (PNW) region led to disease not only in otherwise healthy humans but also domestic, terrestrial and sea animals including dolphins (Stephen *et al.*, 2002, Kidd *et al.*, 2004, MacDougall *et al.*, 2007, Upton *et al.*, 2007). During 1999–2007 the outbreak affected 218 people (5.8 people per million in the region per year) and 19 died (8.7% associated deaths; (Galanis *et al.*, 2010, Phillips *et al.*, 2015)). In addition, during 2004-2011 the outbreak affected approximately 100 people, with a 33% mortality rate, in the US Pacific Northwest (Harris *et al.*, 2011). Most people suffered from respiratory illness (76.6%) or lung cryptococcomas (75.4%; (Galanis *et al.*, 2010)), with a third of those patients also showing central nervous system infection (Phillips *et al.*, 2015).

97% of documented cryptococcosis cases from the Vancouver Island outbreak were compromised of molecular genotype VGII (VG=variety *gattii*; (Boekhout *et al.*, 2001, Kidd *et al.*, 2004)), with subtype VGIIa being responsible for 86.3% of cases in BC (Galanis *et al.*, 2010) and 81% in Washington/Oregon (Harris *et al.*, 2011). The remaining cases were caused by a closely related lineage, VGIIb (also termed the minor lineage; (Kidd *et al.*, 2004)), while a third lineage, VGIIc (novel) has been reported mainly from Oregon State (Harris *et al.*, 2011). VGIIa exhibits higher fertility than other *C. gattii* strains (Ngamskulrungroj *et al.*, 2008) and the molecular type is now considered as the most virulent within this species (Lizarazo *et al.*, 2014).

In comparison to non-outbreak isolates from Australia, where *C. gattii* is endemic, the majority of the Vancouver Island outbreak isolates were highly fertile (70% in BC versus 10% in Australia; (Fraser *et al.*, 2003)), hypervirulent

(Fraser *et al.*, 2005) and showed low susceptibility to anti-fungal drugs (Trilles *et al.*, 2012). Although detailed analysis of many C*. gattii* isolates has been performed, no individual cryptococcal pathogenicity factors have yet been found (Ma *et al.*, 2009), leading to the suggestion that virulence is a complex, multifactorial phenotype (Garcia-Solache *et al.*, 2013, Firacative *et al.*, 2014). Below we discuss some of the features of this pathogen which may contribute to this multifactorial pathogenicity.

Environmental niche of *C. gattii*

While *C. neoformans* spores can be found in birds' droppings, the environmental presence of *C. gattii* is strictly associated with plants. To date 54 species of trees growing globally (Hagen & Boekhout, 2010, Springer & Chaturvedi, 2010) have been found to host *C. gattii*, with Australian eucalyptus (Ellis & Pfeiffer, 1990), almond trees (Callejas *et al.*, 1998) in tropical and semi-tropical regions and, quite recently, *Pseudotsuga menziesii* (Oregon pine) in regions with a more moderate climate (Springer & Chaturvedi, 2010) acting as dominant host species. *C. gattii* can proliferate and mate on plant surfaces rich in myo-inositol (Xue *et al.*, 2007, Springer *et al.*, 2010) and there is some evidence that *C. gattii* may persist longer in the environment in the presence of plant tissue (Huerfano *et al.*, 2001).

The origin of the Pacific Northwest Outbreak

Early investigations proposed several sources for the VGII outbreak strain, but more recent work points to an origin in South America (VGIIa; (Hagen *et al.*, 2013, Billmyre *et al.*, 2014, Engelthaler *et al.*, 2014)) and Australia (VGIIb; (Fraser *et al.*, 2005, Billmyre *et al.*, 2014)). Separation of the VGI and VGII *C. gattii* strains occurred around 12.4 million years ago (D'Souza *et al.*, 2011), and this extended period of genetic isolation has contributed to recent suggestions to raise these lineages to species level (Hagen *et al.*, 2015). Although the molecular genotype of the VGII population differs significantly from other strains of *C. gattii*, due to mutations and recombinations, within the VGII group, the four subpopulations VGIIa, VGIIb, VGIIc and VGIIx are highly clonal and not very

diverse (Billmyre *et al.*, 2014, Engelthaler *et al.*, 2014, Farrer *et al.*, 2015). A comparison between genomes from VGIIa isolates suggests that these groups diverged less than 100 years ago from a less virulent strain in which a frameshift mutation in a DNA repair gene *msh2* was found (Billmyre *et al.*, 2014). VGIIa genomes from the outbreak do not contain the frameshift mutation and it has been hypothesized that its genome reverted via mitotic microevolution (Billmyre *et al.*, 2014). If this is true, there is a possibility that the reversion occurred after gaining adaptation for higher virulence. Since the other non-pathogenic VGIIa-like isolates have retained this hypermutator mutation, it remains possible that such an event may recur leading to the emergence of novel outbreak strains (Billmyre *et al.*, 2014).

In addition, several studies have highlighted the potential for gene transfer (introgression) occurring between *C. neoformans var. grubii* and *C. gattii* (Engelthaler *et al.*, 2014) as well as between different *C. gattii* clades (Billmyre *et al.*, 2014) through sexual reproduction (bisexual or unisexual). Support for the latter model comes from evidence for genomic islands of high polymorphism within a VGIIa genome, which were potentially introduced from two distinct VGII clades (Billmyre *et al.*, 2014).

To date, all clonal *C. gattii* VGII isolates identified from the PNW have been mating type MATα (Lockhart *et al.*, 2013). Unlike MAT**a** strains, MATα strains are capable of same-sex mating (Wiesner *et al.*, 2012) and this has been proposed as a potential origin for the PNW outbreak (Fraser *et al.*, 2005). In *C. neoformans*, MATα strains are also associated with higher virulence (Kwon-Chung *et al.*, 1992, Barchiesi *et al.*, 2005, Nielsen *et al.*, 2005), but this appears not to be the case with *C. gattii* (Zhu *et al.*, 2013).

Genomic differences

Separation between the VGI and VGII *C. gattii* strains has resulted in significant genome differences including chromosomal rearrangements and higher than expected overall nucleotide sequence divergence (D'Souza *et al.*, 2011). Recently a whole genome analysis was performed for all lineages of *C.*

gattii (Farrer *et al.*, 2015), where the authors compared nuclear and mitochondrial DNAs between lineages. Interestingly, they found that the PNW outbreak VGII lost 146 genes (three times more than number of genes lost in VGI-III-IV lineages combined) that are still present in the three other lineages, including a mitochondrial cytochrome c peroxidase gene and several other genes that are typically thought of as being essential for nuclear and mitochondrial genome maintenance. At the same time, the VGII lineage has gained several unique genes encoding proteins with COX6B, HSP70 and iron-binding domains and proteins possibly involved in membrane trafficking.

Lack of RNA interference machinery

One of the most remarkable discoveries to emerge from the extensive genome sequencing effort in this species is the VGII-specific absence of genes encoding Argonaute, Ago1 and Ago2 (D'Souza *et al.*, 2011), which are critical components of the RNA interference (RNAi) machinery in other fungi including *C. neoformans* (Janbon *et al.*, 2010). This lack of *ago* genes was found in all VGII isolates, including those from beyond the PNW region (Farrer *et al.*, 2015). Thus the *C. gattii* VGII lineage is lacking an RNAi-mediated genome defense during both the sexual cycle (Wang *et al.*, 2010, Dumesic *et al.*, 2013) and vegetative growth (Wang *et al.*, 2012). This loss of silencing machinery appears to have independently occurred in several pathogens (reviewed in (Nicolas *et al.*, 2013)), a finding which remains enigmatic. However, this loss of RNAi typically leads to transposon reactivation, which may accelerate genome evolution and potentially help in developing novel anti-host mechanisms (Oliver & Greene, 2009, Biemont, 2010, Wang *et al.*, 2010). Alternatively, the loss of RNAi may be a protective response to pathogens that can otherwise hijack this pathway. Cross-kingdom hijacking of RNAi silencing is known for other pathogens, such as the plant fungal pathogen *Botrytis cinerea* which is able to hijack *Arabidopsis* and tomato RNAi machineries by binding to host AGO1, leading to the silencing of host immunity genes and facilitating infection (Weiberg *et al.*, 2013). To date, pathogens of Cryptococci have not been identified, but such a hypothesis remains at least a theoretical possibility.

Fertility

Although Cryptococci can reproduce sexually, where two opposite mating types, MAT**a** and MAT**α**, mate and produce spores, a predominance of **α** mating type in the environment means that alternative reproduction strategies are common. In particular, same-sex mating between two α mating-type parents (Lin *et al.*, 2005), or spontaneous generation of spores by haploid strains (known as monokaryotic fruiting) can both produce infectious propagules. It has been suggested that the rapid expansion of the PNW outbreak has been driven primarily by clonal reproduction (Fraser *et al.*, 2005) and it is therefore enigmatic that the majority of the PNW outbreak isolates are highly fertile (Fraser *et al.*, 2003, Ngamskulrungroj *et al.*, 2008). Despite this, whole genome analysis has revealed very limited nuclear genetic exchange between *C. gattii* lineages (Farrer *et al.*, 2015), although interestingly several instances of recombination within the mitochondrial genome (Voelz *et al.*, 2013, Farrer *et al.*, 2015).

Inflammation and the cytokine response

In contrast to *C. neoformans*, *C. gattii* is able to infect immunocompetent individuals, suggesting that the latter uses different or additional methods to inhibit immune responses. Somewhat counterintuitively, in human peripheral blood mononuclear cells *C. gattii* induces higher concentrations of cytokines such as pro-inflammatory interleukin IL-1β, TNF- α and IL-6 and the T-cell cytokines IL-17 and IL-22 than *C. neoformans* (Schoffelen *et al.*, 2013). Interestingly, however, the authors found that Toll-like receptor (TLR) 4 and TLR9 were involved in the recognition of the pathogen, but not TLR2, unlike *C. neoformans* (Vecchiarelli, 2005). These results suggested that a different innate cytokine response of the host might be related to different pathogen-activated molecular pattern (PAMPS) molecules localized on the *C. gattii* surface in comparison to *C. neoformans*. TLR2 is known to recognize chitin (Da Silva *et al.*, 2008). Chitin-like structures in *Cryptococci* are only exposed in the limited parts of the cell surface under the capsule (Rodrigues *et al.*, 2008), which may be the reason why they are not normally recognized by TLR2. Thus it is possible that

differences in the organization and localization of chitin-derived structures between *C. neoformans* and PNW *C. gattii* strains might explain different preferences in organ colonization, since *C. gattii* preferentially targets the lungs, whilst the brain is the primary target organ for *C. neoformans* (Ngamskulrungroj *et al.*, 2012, Sorrell *et al.*, 2015).

Although in vitro blood infections by *C. gattii* result in potent inflammatory signalling, in pulmonary tissue Hoang *et al*. found only minimal inflammatory responses to *C. gattii* (Hoang *et al.*, 2004). This may be accounted for by the ability of *C. gattii* to weaken pulmonary Th1 and Th17 responses (at least in mice) via altered dendritic cell (DC) function through down-regulation of pulmonary chemokine expression (Angkasekwinai *et al.*, 2014). This restricted DC function is related to reduced levels of TNF-α, and indeed addition of recombinant TNF-α fully restores DC maturation and thus T cell responses (Huston *et al.*, 2013).

Thus acute introduction of *C. gattii* may induce rapid inflammation, but longer-lasting systemic inflammation is dampened by poor dendritic cell activation. This biphasic response may also explain otherwise contradictory findings, such as the relatively slower growth of *C. gattii* than *C. neoformans* in blood (Ngamskulrungroj *et al.*, 2012) (suggesting strong induction of defense) and yet reduced neutrophil infiltration to sites of infection (Cheng *et al.*, 2009).

Virulence strategies

Human fungal pathogens often use a huge repertoire of virulence strategies in order to survive inside the host. In cryptococci, the polysaccharide capsule, chitin and melanin within the cell wall, phospholipases, urease, laccase and the ability to growth at 37°C are the most studied virulence factors involved in pathogenesis. However, although these features are shared by all pathogenic Cryptococci, there are some crucial differences among them that might play a role in the hypervirulence of PNW *C. gattii* isolates.

Growth at 37°C

The ability to survive at elevated temperature is crucial for human pathogens. In Cryptococci this is regulated by Calcineurin, a Ca^{2+}/calmodulin-activated serine/threonine-specific phosphatase (Liu *et al.*, 1991). Typically, mutants lacking calcineurin gene, such as *cna1Δ*, are avirulent in both *C. neoformans* and *C. gattii* isolates. However, PNW VGIIa strains lacking calcineurin function are still viable at elevated temperature (Chen *et al.*, 2013), suggesting there may be as-yet unidentified differences in temperature tolerance in this lineage.

Capsule

Cryptococcal capsule is a highly hydrated and a negatively charged mesh of polysaccharides surrounding the yeast cell (Fig. 2), mainly composed of glucuronoxylomannan (GXM; composed of mannose, xylose and glucuronic acid), and glucuronoxylomannogalactan (GXMGal) plus mannoproteins (Vartivarian *et al.*, 1989), and its growth is activated during host infection. Most studies to date have focused on *C. neoformans* capsule, which is considered a major virulence factor (McClelland *et al.*, 2006) and has antiphagocytic properties in macrophages (Kozel & Mastroianni, 1976). This is correlated with a reduction of systemic inflammation (reviewed in (Vecchiarelli *et al.*, 2013)) mainly due to a suppression of T lymphocyte proliferation (Syme *et al.*, 1999), induced secretion of the anti-inflammatory cytokine IL-10 (Vecchiarelli *et al.*, 1996) and inhibited secretion of TNF-α and IL-1β (Vecchiarelli *et al.*, 1995) by human monocytes.

GXM is a large molecule (around 4,600,000 Daltons in serotype B strain I23) and has different structures (McFadden *et al.*, 2006) which correlate with differences in antibody reactivity (Fonseca *et al.*, 2010). Capsule size depends on environmental conditions (summarized in (Zaragoza & Casadevall, 2004, Gupta & Fries, 2010)) and capsule enlargement is usually observed during infection (Garcia-Hermoso *et al.*, 2004). This is linked to the presence of mammalian serum (Zaragoza *et al.*, 2003), higher CO_2 concentration (Granger *et al.*, 1985) and tissue-specific conditions such as iron deficiency in the lungs (Vartivarian *et al.*, 1993) (Rivera *et al.*, 1998) or the high concentration of urea in cerebrospinal fluid (Frazzitta *et al.*, 2013). In addition, capsular size can change during the cell

cycle and its enlargement is mainly observed during the G_1 phase when no budding occurs (Garcia-Rodas *et al.*, 2014).

Depending on the *C. gattii* strain, age of the cells, temperature, conditions and methodology used for studies, capsule thickness can differ dramatically. For instance, relative to the canonical *C. neoformans* strain H99 (McFadden *et al.*, 2006), *C. gattii* capsules can be very similar (NIH191 and NIH198 (Frases *et al.*, 2009)), much smaller (strains CN23/10.993 and the PNW strain R265 (Cheng *et al.*, 2009, Fonseca *et al.*, 2010) or significantly larger (strain I23 and R265; (Frazzitta *et al.*, 2013)).

Although the major capsular polysaccharide GXM is generally immunosuppressive, fractions with molecular masses below 10,000 Daltons isolated from *C. gattii* strains were effective in stimulating nitric oxide (NO) production by host macrophages and in activation of TLRs (TLR2/1 and TLR2/6) and NF-κB (Fonseca *et al.*, 2010). Increased production of NO has also been observed after incorporation of extracellular vesicles (EVs) by murine macrophages, but it was diminished after adding fractions of GXM (Oliveira *et al.*, 2010), suggesting that these two components may act in concert to reduce host inflammatory responses.

Interestingly, the cryptococcal capsule is also likely to play an important role in the environment. On plant surfaces, some strains of *C. gattii* form 40–100 nm length extracellular fibrils which then allow yeast cells to escape from human neutrophils in vivo, potentially by inhibiting the production of neutrophil extracellular traps (Rocha *et al.*, 2015). Consequently, infection of mice with yeast cells grown on leaf agar was more severe and showed higher proliferation in the lung and brain then when yeast cells grown on YPD agar were used (Springer *et al.*, 2010).

Extracellular vesicles

GXM, the main polysaccharide component of the cryptococcal capsule, is synthesized intracellularly and transferred from the Golgi apparatus (Hu *et al.*, 2007) to the outside of the cell wall via EVs (Yoneda & Doering, 2006). The

bilayered membrane-EVs serve not only as transporting ports for capsule components, but also are used by cryptococci as 20 to 400 nm diameter "virulence bags" (Rodrigues *et al.*, 2007, Rodrigues *et al.*, 2008). Studies performed so far on *C. neoformans* revealed that EVs contain ribosomal proteins as well as proteins related to virulence and anti-oxidant defense, including laccase (melanin synthesis), urease, superoxide dismutase and heat shock proteins ((Rodrigues *et al.*, 2008) and reviewed in (Rodrigues *et al.*, 2014)). Interestingly, a *C. neoformans sec6* RNAi mutant, which is impaired in EV secretion, was attenuated in virulence in mice, although growth at 37°C, capsule formation and phospholipase activity were not affected (Panepinto *et al.*, 2009). Recent studies on EVs from different fungi including *C. neoformans* revealed that these vesicles are packed with a spectrum of short non-coding mRNAs, which are thought to play a role in cell communication and pathogenesis (Peres da Silva *et al.*, 2015). Unfortunately there is no data regarding function and content of EVs isolated from *C. gattii* to date.

On the other hand, cryptococcal EVs can enhance host antimicrobial activity after incorporation by murine macrophages where increased levels of NO and cytokines (extracellular TNF-α, IL-10, and transforming growth factor (TGF-β)) were observed (Oliveira *et al.*, 2010). Similar results were obtained after treatment of macrophages with EVs isolated from *Candida albicans* (Vargas *et al.*, 2015) suggesting that EVs can serve as a platform of secreted virulence for pathogenic fungi.

At first glance, enhancing host phagocytosis in this way seems like a disadvantageous step for a pathogen. However, since *C. gattii* can happily survive within the phagosome and, at the same time, be protected from other immune cells as well as extracellular antifungal molecules such as complement, it may be that EV-induced boosting of phagocytosis offers survival advantages to pathogens such as *C. neoformans* and *C. albicans* by facilitating their entry into an intracellular niche (Oliveira *et al.*, 2010, Vargas *et al.*, 2015).

Survival within macrophages

Although Cryptococci are phagocytosed by macrophages, in most cases they can then survive and proliferate inside these host cells. Cryptococci have developed an amazing repertoire of anti-phagocytic strategies (reviewed in (Johnston & May, 2013)), most probably as a result of prolonged selective pressure from environmental predators such as amoebae (Steenbergen *et al.*, 2001). As a result, virulence and defense mechanisms against phagocytic cells could be acquired and selected during the evolution of fungus-amoebal interactions in the environment. In support of this model, transcriptional profiles show strong similarities between genes upregulated by yeast internalized by amoebae and murine macrophages (Derengowski Lda *et al.*, 2013). Interestingly, however, relative to *C. neoformans*, *C. gattii* is rarely phagocytosed by the model amoeba *A. castellanii*, perhaps reflecting differences in their capsule structure (Malliaris *et al.*, 2004).

Fungal persistence and reactivation

C. neoformans is classically thought of as a long-term latent pathogen that reactivates upon immunocompromisation, but this picture is more complex for *C. gattii*. Recent multilocus sequence typing between European and worldwide isolates has revealed that dormant *C. gattii* infections can be reactivated many years after the initial infection (Hagen *et al.*, 2012), for instance following treatment with corticosteroids (Hagen *et al.*, 2010). However, unlike *C. neoformans* it appears that many C. *gattii* infections represent de novo acquisition of the organism from the environment, rather than (re)activation of latent disease (MacDougall & Fyfe, 2006).

Proliferation inside macrophages and ''Division of Labour''

All pathogenic Cryptococci appear capable of survival and proliferation within macrophages. However, outbreak *C. gattii* isolates are capable of intracellular proliferation rates that exceed those of all other isolates and which correlate with virulence (Ma *et al.*, 2009). In contrast, *C. neoformans* virulence is associated to macrophage uptake and laccase activity, but not to intracellular proliferation rate (IPR; (Sabiiti *et al.*, 2014)). This difference offers a potential explanation for their varying host profiles; *C. gattii* infections in otherwise

healthy individuals can only proceed if intracellular proliferation is rapid enough to overwhelm the host immune system. In contrast, *C. neoformans* infections in immunocompromised hosts instead rely on "stealth", in which rapid proliferation is not necessarily beneficial but an intracellular niche is critical for survival.

In the case of *C. gattii* outbreak isolates, rapid intracellular proliferation is associated with changes in mitochondrial morphology (Ma *et al.*, 2009, Voelz *et al.*, 2014). It was initially proposed that this change in mitochondrial morphology could protect the pathogen against the intracellular environment of the phagocytic cells (Ma *et al.*, 2009, Ma & May, 2010). However, more recent analyses of this group have indicated a more complex and intriguing model. Upon entry into host phagocytes PNW outbreak strains of *C. gattii* trigger a "Division of Labour" mechanism in which some cells adopt this mitochondrial morphology and cease division, but in doing so they facilitate extremely rapid proliferation of neighboring Cryptococci, thus driving amplification of the population as a whole (Fig. 3; (Voelz *et al.*, 2014)).

Surprisingly, a comparison of mitochondrial genomes between *C. gattii* and *C. neoformans* revealed similar gene content (D'Souza *et al.*, 2011). Likewise, a very recent whole genome analysis did not identify any single gene that is characteristic of the PNW strains (Farrer *et al.*, 2015). However, several studies have highlighted unusual patterns of mitochondrial inheritance and recombination in this lineage (Bovers *et al.*, 2009, Xu *et al.*, 2009, Voelz *et al.*, 2013), suggesting that unusual combinations of nuclear and mitochondrial alleles may contribute to the virulence of this group.

Interestingly, the inheritance patterns of mitochondria in *C. gattii* can be influenced by several environmental variables including UV exposure and higher temperatures (Wang *et al.*, 2015), suggesting an intriguing link between environmental conditions and the evolution of novel genotypes in this group.

Escaping

In addition to intracellular proliferation, *Cryptococci* can also escape from host cells in a poorly understood process called vomocytosis (Alvarez & Casadevall, 2006, Ma *et al.*, 2006). Interestingly, the frequency of this non-lytic expulsion process *in vivo* seems to be higher than the rates obtained *in vitro* (Nicola *et al.*, 2011). There is considerable interest in the contribution that vomocytosis may make to tissue dissemination by allowing infected phagocytes to "deposit" Cryptococci at distant sites; the so-called "Trojan Horse" model. Charlier and colleagues have previously provided evidence for this mechanism of entry across the blood-brain barrier (Charlier *et al.*, 2009), although recent work using *C. neoformans* mutants with reduced phagocytosis by macrophages showed no difference in rates of CNS entry (Tseng *et al.*, 2012). Both *C. neoformans* and *C. gattii* undergo vomocytosis *in vitro*, and rates appear similar, at least between *C. neoformans* H99 and *C. gattii* R265 (Voelz *et al.*, 2009) suggesting that differential vomocytosis is unlikely to be a major factor in PNW virulence. Rather, it appears that the slower growth of PNW *C. gattii* isolates in blood (10-100 times slower than *C. neoformans*) coupled with their exceptionally fast replication within host cells means that *C. gattii* infections frequently present as pulmonary infections rather than disseminated CNS disease (Ngamskulrungroj *et al.*, 2012, Sorrell *et al.*, 2015).

Cell gigantism

Enlargement of the cryptococcal capsule has been documented as a mechanism of protection against phagocytosis and the phagocytic oxidative burst for many years (Zaragoza *et al.*, 2008). However, recently an additional role for cell size increase has become apparent. During in vivo infections, giant or 'titan' cells (50-100 μm in diameter) form and represent about 20% of the cryptococcal population during pulmonary infection (Okagaki *et al.*, 2010, Zaragoza *et al.*, 2010). Intriguingly, the presence of titan cells in the cryptococcal population reduces overall phagocytosis (not just of the titan cells themselves) by macrophages (Okagaki & Nielsen, 2012). Recent studies using the moth larvae *G. mellonella* showed that during *C. gattii* infections, both the capsule and the cell sizes of VGII cells underwent significant enlargements up to 75 μm, but this was not observed in a very virulent PNW isolate R265 (Firacative *et al.*, 2014). These

observations are consistent with a suggestion that cell 'titanisation' provides an additional defense mechanism of the isolates attenuated in virulence (Evans *et al.*, 2015) and/or that this strategy is critical for long-term latent infections, but perhaps less vital for highly virulent acute infections caused by PNW strains.

Conclusions

Recent epidemiological data indicate that the Pacific Northwest outbreak of *C. gattii* infection is receding, although the fungus now appears endemic to Vancouver Island (Espinel-Ingroff & Kidd, 2015, Kwon-Chung & Saijo, 2015), However, understanding the apparently recent and dramatic evolutionary history of virulent VGIIa isolates is of profound importance both for improving our understanding of fungal pathogenesis (Fig. 4) in general and for determining the likelihood of other such outbreaks in the near future.

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Figure Legends

Fig. 1. A schematic illustration of an infection process of *C. gattii* (left) and *C. neoformans* (right). An infection starts upon inhalation of airborne infectious propagules, which may allow the pathogen to settle in the lungs. If the fungus reaches the central nervous system, this can lead to a brain infection, which can be lethal. Note the differences between *C. neoformans* and *C. gattii* environmental origin, the immune condition of the hosts and organ preference between pathogens.

Fig. 2. Diagram representing the role of cryptococcal polysaccharide capsule and its involvement in several immune responses. Chitin-like structures (not shown) composed of β-1,4-N-acetylglucosamine oligomers link

the capsule to the cell wall (Rodrigues *et al.*, 2008). GXMGal molecules (shown in red) are mainly found in growing capsules of budding daughter cell (De Jesus *et al.*, 2009) and also in the capsules of mature cells but only transiently due to secretion (De Jesus *et al.*, 2010). GXM, glucuronoxylomannan; GXMGal, glucuronoxylomannogalactan; EVs, extracellular vesicles.

Fig. 3. Diagram representing a scheme of 'Division of Labour' during engulfment of cryptococcal cell by a macrophage. A) Receptor-mediated phagocytosis allows recognition of the fungal cell **B)** Phagocytosis of the pathogen cell by an alveolar macrophage **C)** One of the first steps of host defense is an oxidative burst during when macrophage releases reactive oxygen species **D)** A subpopulation of guardian cells sacrifice their proliferation and tubularize their mitochondria which is accompanied with reduction of host ROS **E)** This allows proliferation of neighboring cryptococcal cells **F)** and following escape from the macrophage.

Fig. 4. A cartoon representing a repertoire of cryptococcal pathogenic activities.

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